Id proteins function as negative regulators of basic helix-loop-helix transcriptional factors that play important roles in cell fate determination. They preferentially associate with ubiquitously expressed E proteins of the basic helix-loop-helix family and prevent them from binding to DNA and activating transcription. Although their small size suggests that Id proteins enter and exit the nucleus by passive diffusion, several studies have indicated that other pathways may regulate their subcellular localization. In this study, we obtained evidence that Id2 has the ability to shuttle between the nucleus and the cytoplasm. When passive diffusion was prevented by fusion with green fluorescent protein (GFP), Id2 was predominantly localized in the cytoplasm. Using GFP fusion constructs, we demonstrated that the C-terminal region is required for cytoplasmic localization. Nuclear accumulation of GFP-Id2 in cells treated with the nuclear export inhibitor leptomycin B suggests that the nuclear export receptor CRM1 mediates the cytoplasmic localization of Id2. Id2 contains two putative leucine-rich nuclear export signals, and the nuclear export signal in the C-terminal region is essential for nuclear export. On the other hand, the helix-loop-helix domain is important for nuclear localization. Finally, experiments using reporter assays revealed an inverse correlation between nuclear export and transcriptional repression via the E-box sequence. Based on all these findings, we propose that nucleo-cytoplasmic shuttling is a novel mechanism for the regulation of Id2 function.

Id proteins function as negative regulators for basic helix-loop-helix transcriptional factors that specifically regulate gene expression during cell fate determination. They play pivotal roles in differentiation, cell cycle control, and cell lineage commitment in both vertebrates and invertebrates (1, 2). Id proteins are involved in the regulation of determination and differentiation of committed progenitors in the hematopoietic lineage (3–5). The functions of Ids are strictly regulated at levels of transcription (6, 7) and protein stability (8). Recent studies have revealed additional roles for these proteins in apoptosis, cellular senescence, and tumorigenesis (2, 9, 10).

All members of the Id protein family share a similar overall structure consisting of the highly conserved helix-loop-helix (HLH) domain and the less conserved N- and C-terminal regions. Via their HLH domain, Id proteins preferentially associate with ubiquitously expressed bHLH E proteins such as E2A, E2–2, and HEB and sequester them from tissue-specific bHLH proteins exemplified by MyoD and Mash1 (1, 2, 11). Because Id proteins lack a basic region that is critical for DNA binding, the heterodimer between Id and E proteins fails to bind to an E-box DNA sequence, and consequently the expression of genes possessing the E-box sequence in their regulatory elements is repressed. The affinities of Id2 and Id3 for E proteins are modulated by cell cycle-dependent phosphorylation of the Ser-5 residues in the N-terminal regions (12, 13). The N-terminal region of Id2 is also important for apoptotic induction (14) and protein degradation (15). However, little is known about the molecular function of the Id protein C-terminal regions.

Appropriate subcellular localization is crucial for the proper function of numerous proteins. Although some proteins are constitutively nuclear, others are actively imported into and exported out of the nucleus, in a signal-dependent or -independent manner (16, 17). Large proteins shuttle between the nucleus and the cytoplasm through nuclear pore complexes by virtue of their intrinsic nuclear localization signals (NLSs) and nuclear export signals (NESs). The NLSs and NESs are recognized by specific import and export receptors, respectively (18, 19). In contrast, small proteins with molecular masses of less than 40 kDa can freely pass through the nuclear membrane (16, 18). Because the four members of the Id protein family have molecular masses ranging from 13 to 18 kDa, they are thought to enter and exit the nucleus by passive diffusion. In fact, small epitope-tagged Id2 protein is detected in both the nuclear and cytoplasmic compartments of transfected COS cells (20).

Passive diffusion seems to be one mechanism deciding the subcellular localization of Id proteins, but it is likely that other regulatory pathways also exist. Norton and co-workers (21) previously showed that transiently expressed Id3 accumulated in the nucleus when one of the E2A gene products, E47, was co-expressed. Hence, they concluded that E protein acts as a nuclear chaperone for Id proteins. On the other hand, Samanta and Kessler (22) have recently presented the opposite findings. Their data suggest that Id1 and Id4 would sequester nuclear bHLH proteins to the cytoplasm to promote differentiation of cultured neural progenitors into astrocytes. Furthermore, the
subcellular localization of one of the Id protein family members, Id2, has been reported to change from the nucleus to the cytoplasm during neural differentiation into oligodendrocytes (23). Nuclear exclusion of Id2 is also observed when myeloid precursors are induced to differentiation (24).

Here we describe the nucleo-cytoplasmic shuttling of Id2. Our results demonstrated that Id2 is predominantly localized in the cytoplasm under conditions in which passive diffusion does not occur. Then we characterize the molecular determinants that govern the predominant cytoplasmic localization and identify a functional leucine-rich NES in the C-terminal region. We also show that Id2 is localized in the nucleus when nuclear export is blocked and that the HLH domain is important for nuclear localization. Finally, our data suggest that nuclear export of Id2 has an inhibitory effect on repression of the E-box-mediated transcription.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—For construction of the N-terminally GFP-tagged Id1–Id4 plasmids, cDNAs encoding full-length mouse Id1–Id4 were amplified with HindIII/BamHI GFP fusion primers (Roche Diagnostics). Deletion and mutant constructs of GFP-tagged Id1–Id4 were generated by PCR amplification using their respective wild-type constructs as templates and appropriate primer pairs including artificial HindIII (for forward primers) and BamHI (for reverse primers) restriction enzyme sites. All of the constructs were verified by DNA sequencing. A tandem-duplicated GFP (GFP × 2) plasmid was constructed by ligating the PCR-amplified BamHI-HindIII fragment of the enhanced green fluorescent protein (EGFP) coding region to the BglII/HindIII sites of pEGFP-P-C1. Two oligonucleotides, 5′-AGCTTGGCTGACCAAGGGAAGCACG-GACATCCACTCTTGTCGACGGGCATCTGAAG-3′ (forward) and 5′-GATCTCTCTAGACCTGCGAGGAGGAGGTGTCGAGCTGGTGTTGACGCA-3′ (reverse), were annealed and inserted into the HindIII/BamHI sites of the GFP × 2, GFP-Id2, and GFP-Id4 plasmids. GFP-Id1-Id4 (103–119) fusion proteins were generated by PCR amplification using their respective primers and the HindIII/BamHI sites of the GFP × 2, GFP-Id1-Id4 (103–119) plasmids. For Western blot analysis, the cell lysates were concentrates using Microcon YM-10 (Millipore) according to the manufacturer’s instructions.

RESULTS

The C-terminal Region of Id2 Is Required for Cytoplasmic Localization—To examine the subcellular localization of Id2, we generated a construct wherein the full-length mouse Id2 was N-terminally tagged with GFP (Fig. 1A). The size of the fusion protein was around 45 kDa (Fig. 1C), and we assumed that it would not undergo passive diffusion. As shown in Fig. 1B, GFP-Id2 was predominantly localized in the cytoplasm when it was transfected into NIH3T3 cells. Similar results were obtained in COS7, 293T, and U2OS cells (data not shown). Unlike GFP-Id2, fluorescently tagged Id3 was previously shown to be diffusely distributed throughout the cells (27). Thus, the cytoplasmic localization of GFP-Id2 is not cell type-dependent and is not a general effect caused by fluorescence with a fluorescence tag. The difference in subcellular localization between fluorescently tagged Id2 and Id3 suggests that the former contains a specific domain or signal that facilitates cytoplasmic localization. Because amino acid sequences outside the HLH domains are poorly conserved among the Id protein family (11), we next examined whether the N- or C-terminal region of Id2 is involved in mediating cytoplasmic localization. Whereas truncation of the N-terminal region had no effect on the subcellular localization, truncation of the C-terminal region resulted in nuclear accumulation of the fusion protein (Fig. 1, A and B). To define the region required for cytoplasmic localization, we generated several C-terminal deletion constructs. Although GFP-Id2(1–101) showed nuclear localization, GFP-Id2(1–119) was located in the cytoplas, indicating that amino acids between residues 102 and 119 are required for cytoplasmic localization. An internal deletion mutant, GFP-Id2(1–119)Δ77, exhibited a cytoplasmic localization pattern indistinguishable from that of GFP-Id2(1–119). These results demonstrate that amino acids between residues 103 and 119 are responsible for the cytoplasmic localization. Protein expression of the fusion constructs was confirmed by Western blot analysis using anti-GFP antibody (Fig. 1C).

Id2 Is Exported from the Nucleus via a CRM1-dependent Mechanism—Remarkably, the 17-amino acid sequence identified above contains a leucine-rich stretch that well matches the regularly spaced hydrophobic residues in the NES consensus sequence (28–30). A similar region is present in Id1 but not in Id3 or Id4 (Fig. 2A). The cytoplasmic localization of NES-containing proteins is due to active nuclear export, which is in many cases mediated by the nuclear export receptor CRM1 (chromosome region maintenance protein 1) (16–19). To ex-
plore the possibility that Id2 is actively exported from the nucleus via a CRM1-dependent mechanism, cells expressing the GFP-Id2 fusion protein were treated with the CRM1-specific nuclear export inhibitor LMB (31, 32). After treatment of the cells with LMB, GFP-Id2 accumulated in the nucleus, and only a small proportion remained in the cytoplasm (Fig. 2B). This change of localization suggests that CRM1 mediates the nuclear export of Id2.

Because some isolated NES sequences are still active in nuclear export when transferred to other proteins (33), we tested whether the putative NES located in the C-terminal region of Id2 is capable of directing a heterologous protein to the cytoplasm. A single GFP moiety is small enough to enter the nucleus by passive diffusion because its size (26 kDa) is below the exclusion limit of nuclear pores. To avoid passive diffusion of the reporter protein, we generated a tandem-duplicated GFP (GFP/H11003_2) construct and connected it to the C-terminal 17-amino acid sequence of Id2. As reported previously (34–37), GFP/H11003_2 was uniformly distributed throughout the cells regardless of whether or not they were treated with LMB (Fig. 2C, left panels). In contrast, the fusion protein of GFP × 2-Id2(103–119) was exclusively localized in the cytoplasm of the untreated cells, and LMB effectively blocked this cytoplasmic localization (right panels). These results clearly show that the putative NES of Id2 is autonomously functional in the CRM1-mediated nuclear export.

The C-terminal NES of Id2 Is Essential for Nuclear Export—Careful examination of the amino acid sequences revealed the existence of a second leucine-rich region that conforms to the NES consensus motif at the end of the Id2 HLH domain. For simplicity, we refer to the NES sequences in the HLH domain and C-terminal region as NES-1 (residues 65–75) and NES-2 (residues 106–115), respectively (Fig. 3A). To evaluate their contribution to nuclear export, we mutated either the second or fourth conserved hydrophobic residue to alanine in the two NES sequences and examined the effect on the subcellular localization of the GFP-Id2 fusion protein. Although neither mutation in the NES-1 sequence (I69A and L75A) affected the cytoplasmic localization of GFP-Id2, both of the mutations in the NES-2 sequence (I110A and L115A) led to nuclear translocation of the fusion protein (Fig. 3B). Therefore, we concluded that the NES-2 in the C-terminal region is essential for nuclear export of Id2.

The HLH Domain of Id2 Is Important for Nuclear Localization—Treatment with LMB or disruption of NES caused GFP-Id2 to be accumulated in the nucleus rather than diffusely distributed throughout the cell. This implies that Id2 may contain a specific domain or signal that promotes nuclear accumulation when nuclear export is blocked. Because GFP-Id2(1–76) comprising the N-terminal and HLH domains was exclusively localized in the nucleus (Fig. 1), we next analyzed the involvement of the short clusters of basic residues located...
The C-terminal 17-amino acid sequence is shown at the bottom, and conserved hydrophobic residues of the aligned sequences are highlighted by boxes. 6 indicates hydrophobic residues including leucine, isoleucine, and valine. B and C, NIH3T3 cells were transiently transfected and cultured for 24 h. Then they were incubated in the absence or presence of LMB (5 ng/ml) for further 3 h. Subcellular localization was examined as described under "Experimental Procedures." The upper and lower panels are green fluorescence and DAPI staining images, respectively. B, representative images of cells transfected with the plasmid DNA encoding GFP-Id2. LMB treatment led to accumulation of GFP-Id2 in the nucleus. C, representative images of cells transfected with a fusion construct between tandem-duplicated GFP and the Id2 C-terminal 17-amino acid sequence that contains a putative NES. The C-terminal 17-amino acid sequence of Id2 has an autonomous nuclear export activity.

Decreased Nuclear Export of Id2 Augments Repression of E-box-mediated Transcription—Regulated nuclear import and export are important for the physiological function of signaling molecules, particularly transcription factors. To investigate whether nucleo-cytoplasmic shuttling of Id2 plays a role in E-box-mediated transcription, we generated GFP-Id2 fusion constructs in which the first and second or the third and fourth conserved residues in the NES-2 sequence were simultaneously mutated (L106A/I110A and L113A/L115A, respectively). When the double mutants were transfected into NIH3T3 cells, they exhibited the same nuclear localization pattern as the single mutants of the NES-2 sequence (Figs. 3 and 5A). We next examined their association with E protein in transfected COS7 cells by co-immunoprecipitation. The wild-type GFP-Id2 was co-precipitated with E47-FLAG (Fig. 5B, lane 7), whereas the HLH domain-deleted mutant GFP-Id2ΔHLH was not (lane 8), ensuring the structural integrity of the fusion protein between GFP and Id2. The double mutations of NES-2 did not affect the interaction with E protein because E47-FLAG co-precipitated GFP-Id2(L106A/I110A) and GFP-Id2(L113A/L115A) as efficiently as GFP-Id2 (lanes 9 and 10 versus lane 7).

We then measured the repression activity of the fusion proteins in transactivation of the E-box-containing reporter achieved by co-expression of MyoD and E47. As shown in Fig. 5C, the wild-type GFP-Id2 protein repressed the MyoD-E47-induced transactivation in a dose-dependent manner (lanes 7–10), as reported in our previous study using the untagged Id2 construct (26). Given that GFP-Id2 is able to shuttle between the nucleus and the cytoplasm, its repression activity is not surprising. One can envisage that GFP-Id2 enters the nucleus...
to repress the MyoD-E47-induced transactivation. In this context, GFP-Id2(L106A/I110A) and GFP-Id2(L113A/L115A) were slightly but significantly more potent at repressing transactivation as compared with the wild type (lanes 11–13 and 14–16). These data suggest that nuclear export of Id2 has an inhibitory effect on its activity to repress transactivation by the bHLH proteins.

The C-terminal NES-mediated Nuclear Export Is Specific to Id2—Id1 possesses the NES-like sequence in the C-terminal region, but the fourth conserved residue, which has been predicted to be most important for NES function (39), is alanine instead of leucine (Fig. 6A). To determine whether the NES-like sequence of Id1 is functional in nuclear export, we generated a double mutant in which the first and second conserved hydrophobic residues were replaced by alanines (L124A/I128A). GFP-Id1, as well as GFP-Id2, was predominantly localized in the cytoplasm when transfected into NIH3T3 cells (Fig. 6B, left panels). The GFP-Id1(L124A/I128A) mutant was also localized in the cytoplasm (right panels), unlike the similar double mutant GFP-Id2 (L106A/I110A), which displayed nuclear localization (Fig. 5A, left panels). This indicates that the NES-like sequence of Id1 is inactive in nuclear export, and other sequences must be involved in mediating the cytoplasmic localization. We then analyzed the effect of the mutation on the repression of E-box-mediated transcription. GFP-Id1 and GFP-Id2(L113A/L115A) showed similar dose-dependent repression of MyoD-E47-induced transactivation (Fig. 6C). Thus, the mutation of the NES-like sequence of Id1 had no effect on either its nuclear export or its transcriptional repression.

Increased Nuclear Export of Id3 and Id4 Reduces Their Repression Activity in E-box-mediated Transcription—In agreement with the previous report (27), GFP-Id3 was distributed throughout the cells, and GFP-Id4 also exhibited diffuse nucleo-cytoplasmic localization (Fig. 7A). To further address the inhibitory effect of nuclear export on transcriptional repression, we added the Id2 NES-2 sequence to the C termini of GFP-Id3 and GFP-Id4 to enforce nuclear exclusion. As expected, the fusion proteins containing the NES were predominantly localized in the cytoplasm (Fig. 7A). We then examined their ability to repress E-box-mediated transcription. At low levels of expression, both of the NES-added fusion proteins...
and the NES-like sequence mutant GFP-Id1 (L124A/L128A). The native images of NIH3T3 cells transiently transfected with the GFP-Id1 arrows. NES consensus sequence is shown at the lighted by boxes. Conserved hydrophobic residues are highlighted by green fluorescence and DAPI-staining images, respectively.

**FIG. 6.** The NES-like sequence in the Id1 C-terminal region is not functional in nuclear export. A, alignment of the Id2 NES-2 and the Id1 NES-like sequences. Conserved hydrophobic residues are highlighted by boxes, and mutated amino acids in Id1 are indicated by arrows. NES consensus sequence is shown at the bottom. B, representative images of NIH3T3 cells transiently transfected with the GFP-Id1 and the NES-like sequence mutant GFP-Id1 (L124A/L128A). The upper and lower panels are green fluorescence and DAPI-staining images, respectively. C, 200 ng of pE7-TK-luc was transiently transfected into NIH3T3 cells with 100 ng of pcMV-MyoD and 200 ng of pcDNA3.1, E7-47 × 2 – FLAG and increasing amounts (10, 30, and 100 ng) of pEGFP-Id1 or pEGFP-Id1L124A/L128A. The error bars indicate S.E. Expression of the GFP-Id1 fusion proteins analyzed by Western blot using anti-GFP antibody was shown at the bottom. WT, wild type.

Repressed MyoD-E47-induced transactivation comparatively with their respective nonadded forms (Fig. 7B, lane 6 versus lane 3 and lane 14 versus lane 11). At high levels of expression, however, the repression activities of the NES-added proteins were less strong than those of their nonadded forms (lanes 7 and 8 versus lanes 4 and 5, and lanes 15 and 16 versus lanes 12 and 13). Collectively, our results suggest that nuclear export of Id proteins plays an inhibitory role in their suppressive activity against bHLH factors.

**DISCUSSION**

In this study, we have demonstrated that Id2 is predominantly localized in the cytoplasm when passive diffusion is prevented because of fusion with GFP. We have also shown that Id2 can be actively exported from the nucleus by a CRM1-dependent mechanism. Id2 contains two putative leucine-rich NESs, NES-1 in the HLH domain and NES-2 in the C-terminal region, and mutational analysis clearly showed that the C-terminal NES-2 is essential for nuclear export. NES-2 is evolutionarily conserved in vertebrate Id2 proteins of species from *Xenopus* (40) to human (41), supporting its functional importance. Notably, NES-2 is characteris-

**tic** of Id2, whereas the NES-1 is conserved among the Id family members (data not shown). Thus, our study revealed a novel function of the Id2 C-terminal region in determining the subcellular localization of Id2. On the other hand, the inhibition of nuclear export led to nuclear accumulation of Id2, without co-expressed E protein. This suggests that Id2 itself has the nuclear localization activity, which is usually overridden by the NES-2 in the C-terminal region. Although the canonical NLS is not found in Id2, our data indicated that basic residues in the HLH domain are critical for nuclear localization. Similar results were recently reported for Id1 (42). To our knowledge, this is the first report describing the nucleo-cytoplasmic shuttling of an Id family member.

Accumulating evidence has demonstrated that the expression of Id proteins is significantly correlated with the progression of cancer, and therefore Id proteins are now considered to be oncogenes (9). Consistent with this notion, expression of Ids is strictly regulated at the transcriptional level (3, 6, 7), and recent studies have shown that protein degradation is one mechanism of their post-translational regulation (8, 15, 42). Because our experiments using reporter assays suggest that nuclear export of Id2 has an inhibitory effect on transcriptional repression, nucleo-cytoplasmic shuttling would be another type of the post-translational regulation of the Id2 function. The importance of nucleo-cytoplasmic shuttling has recently emerged for several oncogenes and tumor suppressors (43, 44). Above all, nucleo-cytoplasmic shuttling of p53 has been extensively analyzed (45).

The present study also suggested that the subcellular localization of different Id protein family members is regulated by different mechanisms. Id1 contains the NES-like sequence in the C-terminal region, but it is not functional in nuclear export, probably because of the substitution of the fourth critical conserved hydrophobic residue. The cytoplasmic localization of GFP-tagged Id1 may depend on the putative NES in the HLH domain, although the Id2 NES-1 in the HLH domain is dispensable for its nuclear export. In contrast to Id1 and Id2, Id3 and Id4 are distributed throughout the cells even when passive diffusion is prevented by fusion with GFP. They appear to lack a functional NES, and their localization inside the cells would be dependent basically on passive diffusion and occasionally on interaction with E protein (21). Such different modes of regulation of subcellular localization may give rise to the functional diversity of the Id protein family members.

Nucleo-cytoplasmic shuttling of proteins is subject to diverse types of regulation. Actually, the subcellular localization of many proteins is regulated by protein modifications such as phosphorylation. In this regard, it was shown that an unphosphorylatable mutant of Id2 at the N-terminal serine residue was localized in the cytoplasm of vascular smooth muscle cells, whereas the wild-type Id2 exhibited nuclear localization (46). Although the data implied that the nucleo-cytoplasmic shuttling of Id2 might also be regulated by N-terminal phosphorylation, the same unphosphorylatable mutation did not affect the localization of GFP-Id2 or its C-terminally truncated form. Nevertheless, it is conceivable that phosphorylation of other regions is important for regulation of the nucleo-cytoplasmic shuttling of Id2. Other types of regulation include masking and unmasking of NESs and NLSs. Association with other proteins sometimes results in direct masking of the target signals or indirect masking or unmasking of them by alteration of protein conformation. Liu et al. (47) reported that an interferon-inducible p200 family protein, p204, interacted with Id2 and negated its inhibitory effect on myogenic differentiation. They postu-
lated that p204 sequesters Id2 from the nucleus to the cytoplasm when myoblasts differentiate into muscle cells (47, 48). Because p204 binds to the HLH domain of Id2, it may suppress the nuclear localization activity of the HLH domain. Alternatively, p204 may prevent Id2 from undergoing passive diffusion by increasing the molecular size. Nuclear exclusion of Id2 observed in oligodendrocytes (23) and myeloid cells (24) could be due to association with other proteins like p204 or modifications of the protein.

It has been thought that Id proteins function in the nucleus to repress transactivation by bHLH proteins, but Samanta and Kessler (22) have recently raised the interesting possibility that Id2 may sequester nuclear bHLH proteins to the cytoplasm. Given that GFP-Id2 is able to shuttle between the nucleus and the cytoplasm, it is possible that the transcriptional repression by GFP-Id2 in our reporter assays is the net result of inhibition of the bHLH protein activity in the nucleus and sequestration of the nuclear bHLH proteins to the cytoplasm, which could account for the smaller difference of repression activities between the NES active and inactive forms than we expected (Figs. 5 and 7). In addition, Id2 may play a role other than as an inhibitor of bHLH proteins. This possibility will be explored by isolation and characterization of Id2-associated molecules in the cytoplasmic fraction of cells.

We examined the subcellular localization of Id2 using GFP-tagged constructs to avoid passive diffusion. This strategy is often used to analyze nucleo-cytoplasmic shuttling of small proteins (35, 49–52). Activation-induced cytidine deaminase is an essential factor for class switch recombination, somatic hypermutation, and gene conversion, which are all nuclear events (53). Using fusion constructs with a fluorescence tag, three independent groups have recently reported that activation-induced cytidine deaminase shuttles between the nucleus and the cytoplasm despite its relatively small size (~24 kDa) (49–51). Most proteins with small molecular masses are thought to enter and exit the nucleus by passive diffusion. However, recent studies including ours suggest that the subcellular localization of some small proteins changes more dynamically than has been expected, in accord with their multiple functions.
Acknowledgments—We are grateful to Y. Matsui for excellent technical assistance. We also thank M. Yoshida for LMB, S. Mori for the GFP-tagged full-length mouse Id2 plasmid, and M. Sugai for the plasmid BSKS(+)E472×2-FLAG.

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Nucleo-cytoplasmic Shuttling of Id2, a Negative Regulator of Basic Helix-Loop-Helix Transcription Factors
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doi: 10.1074/jbc.M412614200 originally published online November 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M412614200

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